American Journal of Pathology, Vol. 142, No. 5, May 1993

SV40 Induces Mesotheliomas in Hamsters

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In the course of studies to elucidate the relative contribution of simian virus 40 (SV40) large T and small t proteins during oncogenesis, we observed the appearance of pericardial and pleural tumors in 100% of Syrian hamsters injected in the pleural space with wild type SV40. When SV40 was injected via the intracardiac or intraperitoneal routes, more than 50% of hamsters developed mesothelial tumors. Macroscopic, microscopic, ultramicroscopic, and histochemical characteristics identify these neoplasms and derived cell lines as mesotheliomas and mesothelioma-derived cell lines. The SV40 genome was integrated and expressed in the mesotheliomas and derived cell lines. The absence of mesotheliomas in hamsters injected with SV40 small t deletion mutants indicates that the small t protein plays an important role in the development of SV40-induced mesotheliomas. To the best of our knowledge, this is the first definitive report of virus-induced mesotheliomas in mammals. (Am J Pathol 1993, 142:1524–1533)

Malignant mesotheliomas are tumors originating in the serosal lining of the pleural cavities. These are relatively rare human tumors; however, an increasing number of cases are anticipated over the next 10 years.[1,2] Although formerly seen predominantly in men employed in high-asbestos-exposure occupations, malignant mesotheliomas are being seen more frequently in women and in those with no history of work-related exposure to asbestos.[1–3] The molecular alterations leading to mesothelioma are still unknown, and many attempts have been made to find an appropriate animal model for this fatal human disease. Mesotheliomas can be induced within about 2 years in 19% to 73% of rodents following intrapleural injections of various forms of asbestos, silica, organic chemicals, and radioactive compounds.[3,4] In addition, an RNA virus, the MC29 avian leukosis virus, has been found to induce mesotheliomas in 35% of chickens injected in the peritoneal, pericardial, and air sac cavities.[5] DNA tumor viruses, including SV40, have not been reported to induce mesotheliomas. However, fetal mice inoculated *in utero* with polyoma virus developed proliferative lesions of the mesothelium. Although these lesions sometimes covered the entire serosa, they did not become malignant and later regressed completely.[6] The problems associated with most of these experimental mesothelioma models include the long latency, the relatively low number of animals developing mesotheliomas,[3,4] and the appearance, in addition to mesotheliomas, of other tumors, such as liposarcomas and rhabdomyosarcomas.[3,4] Thus, the availability of an animal model in which mesotheliomas could be induced in 100% of the animals within a few months would be very useful.

The SV40-hamster tumor system has been widely used as a model for understanding human carcinogenesis. Wild type (wt) SV40 is highly oncogenic in hamsters. Newborn animals are particularly susceptible, usually developing fibrosarcomas at the injection site following subcutaneous inoculation of a low dose of SV40.[7] When newborn hamsters are inoculated with SV40 intracerebrally they develop ependymomas.[8] Weanling and adult animals may develop fibrosarcomas if injected subcutaneously with a high dose of virus[9] [$>10^9$ plaque-forming units (pfu)]. When SV40 ($>10^{8.5}$ pfu) is injected intravenously into weanling hamsters, a circumstance in which many cell types are exposed to high concentrations of the virus, lymphocytic leukemia, lymphoma, soft tissue sarcoma, and/or osteosarcoma occur.[10]

The recent discovery of an association of the large T antigen of SV40 with both the retinoblastoma (RB) and p53 cellular gene products and the clear correlation of defects in these same cellular proteins (RB and p53) with a number of human tumors strongly suggest that the transformation of cells by SV40 may have direct relevance to our understanding about the molecular events that lead to cancer in man. The

Accepted for publication November 20, 1992.

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SV40 gene products responsible for its transforming functions, the large T antigen (90 kd) and the small t antigen (19 kd), are encoded by the early region of the viral genome. The large T antigen is needed for both cell transformation and oncogenicity,[11–13] whereas the exact role of the small t antigen in SV40 oncogenesis is still unclear.[14–16] In this study, we investigated the oncogenicity of wt SV40 and SV40 small t deletion mutants (dl) when injected intracardiacally (ic), intrapleurally, (ipl) and intraperitoneally (ipt) into weanling hamsters (21 days old). To the best of our knowledge, the oncogenicity of SV40 using these routes of injection has not been previously explored.

Materials and Methods

Viruses

SV40 and its mutant derivatives were propagated in the TC-7 clone of the CV1 African green monkey kidney cell line. Two wt strains (wt 830 and wt 776) and two small t mutants (dl 883 and dl 2006) were used in these studies. The wt 830 is the parent virus of dl 883,[17] which has a 57-bp deletion that spans the small t donor splice site.[18] The dl 2006 mutant is derived from the wt strain 776[19] and contains a slightly larger deletion of small t antigen sequences (250 bp); however, the deletion does not extend into the small t intron. Virus stocks were grown by infecting subconfluent (50%) TC-7 cells at a multiplicity of infection of 0.05 in 175-cm^2 tissue culture flasks in the presence of 30 ml of Dulbecco's minimum essential medium (DMEM) with 5% fetal bovine serum (FBS). Viruses were purified through sucrose gradients by the method of Rosenberg et al.[20] Virus preparation was under strictly sterile conditions, and virus titration was determined by plaque assay. Concentrations of the infectious virus varied from 1 $\times$ 10^9 to 6 $\times$ 10^9 pfu/ml.

Experimental Design

We injected ic 21-day-old Syrian hamsters (50% males, 50% females) with wt SV40 830 (11 hamsters injected), SV40 small t mutant dl 883 (six hamsters), SV40 small t mutant dl 2006 (10 hamsters), and media alone (the media in which the viruses were diluted: DMEM and 2% FBS, control group, 10 hamsters). After a period of 9 months, based on the results obtained in this first set of experiments, a second group of hamsters were injected with wt SV40 830 (10 hamsters injected ic and six injected ipl), wt SV40 776 (five hamsters ic, five ipl, and six ipt), SV40 small t mutant dl 883 (six hamsters ic), SV40 small t mutant dl 2006 (12 hamsters ic), and media alone (control group, 10 hamsters ic, four ipl, and four ipt). The results of these experiments are presented together.

Animal Injection

Hamsters (kept under sterile conditions throughout the 9 months of the experiment) were anesthetized with an ipt injection of 0.1 to 0.3 ml of a solution containing ketamine and xylazine and injected with $10^{8.5}$ pfu of purified virus (diluted in DMEM and 2% FBS) in the left cardiac ventricle using a microinfusion set containing a 25-gauge cannula. For ic injections, the needle was inserted into the intercostal space, and when pulsating oxygenated blood appeared in the plastic tube, the virus was injected. After the injection, the blood color and pulsation were again checked to ensure that the virus was correctly injected into the left cardiac ventricle. For ipl injections, two different needles were used: the first to insert air in the pleural space and collapse the lung and the second to inject the virus. For peritoneal injections, the viruses were injected directly into the peritoneal cavity. The volume injected ic, ipl, and ipt was 0.2 ml. Injected animals were observed twice a week for tumor formation. Tumor-bearing animals appearing ill were killed with CO_2 and necropsied under aseptic conditions. All animals were sacrificed by the end of 9 months. Samples from brain, heart, lungs, kidneys, spleen, abdominal lymph nodes, and, when indicated, bones and muscle tissue, were histologically examined for detection of microscopic tumors in all of the animals studied, including the control group.

Establishment of Tumor Cells in Culture

Tumor cell lines were established from 17 mesotheliomas by mincing tumor tissue and allowing the cells to grow in plastic tissue culture flasks in a 1:1 mixture of DMEM (GIBCO-Bethesda Research Laboratories, Gaithersburg, MD) and FT12 (GIBCO-Bethesda Research Laboratories) containing 10% FBS, 2 mmol/L glutamine, 1% penicillin-streptomycin, and 1% gentamicin. This particular mixture of media is recommended by GIBCO-Bethesda Research Laboratories to support the growth of most cell types. All of the cell lines were studied between passage 1 and 10.

Electron Microscopy

The cells and/or tissue were fixed by immersion in 2.5% glutaraldahyde (Tousimis Research, Rockville

MD) in Millonig's sodium phosphate buffer for 2 hours at 4 C. The specimens were cut into small pieces no larger than 1 mm^3 and postfixed in 1% osmium tetroxide (Polyscience Inc., Warrington, PA) for 2 hours at 4 C. The specimens were en bloc stained with uranil acetate, dehydrated in a series of ethanols, infiltrated, and embedded in Spurr's plastic resin.[21] After polymerization, the blocks were cut using the Reichert-Jung Ultracut E ultramicrotome, and sections 60 to 80 nm in thickness were collected on copper grids and poststained with lead-citrate. The grids were thoroughly examined in a Hitachi HU-12A transmission electron microscope.

DNA Extraction and Southern Blot Analysis

Genomic DNA from hamster tumors was prepared by the sodium dodecyl sulfate–proteinase K procedure[22] and digested by various restriction enzymes. After agarose gel electrophoresis, DNA was transferred to a nylon membrane (Gene Screen Plus, NEN, Boston, MA) by capillarity and UV cross-linked using the UV-Stratalinker (Stratagene, La Jolla, CA). The DNAs were hybridized to a nick-translated ^{32}P-labeled probe by overnight incubation with 3×10^6 cpm of ^{32}P-labeled probe per ml of hybridization buffer. The final wash was at 65C, 0.1× standard saline citrate. Filters were dried briefly and exposed to x-ray film.

Immunoprecipitation and Immunohistochemistry

Preparation of radiolabeled cell lysates was as previously described.[16] Briefly, lysates were labeled with 300 uCi of [^{35}S]methionine. Samples (300 ug) from each cell lysate were immunoprecipitated with the monoclonal antibody SV40 Tag (Ab-1) clone PAb 419 (Oncogene Science, Manhasset, NY), or with an antiserum obtained from SV40 tumor–bearing hamsters. The resulting immunocomplexes were analyzed by 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions, followed by fluorography. The immunofluorescence assays used to detect the presence of SV40 T antigen in tumor cells were as described.[14] To investigate the presence of cytokeratins, we used a rabbit polyclonal antibody (BioGenex, San Ramon, CA). This antibody was chosen because according to the manufacturer it cross-reacts with cytokeratins from various species, including hamsters. The reaction was visualized by immunoperoxidase using a commercially available kit (BioGenex).

Results

Macroscopic Characteristics of SV40-Induced Tumors

Approximately 3 months after injection, hamsters injected ic or ipl with wt SV40 suddenly developed dyspnea, appeared lethargic, and became extremely ill. Upon necroscopy, these animals revealed extensive tumor formation within the thorax. These tumors formed a continuous layer over the pleural and the pericardial surfaces, encasing the lungs and often the heart and obliterating the pleural and pericardial cavities. Multiple gray or white ill-defined nodules were seen in a diffusely thickened pleura. Pleural effusion was always present. The tumors did not penetrate deeply into the lung or heart tissue; however, they spread extensively over the chest wall and the diaphragm, which were often infiltrated. No distant metastases were observed. Some of the hamsters injected ipt with wt SV40 developed lesions similar to those described above but in the peritoneal space. These primary peritoneal tumors spread widely over the serosal surfaces, including that of the intestine, and were easily distinguished from secondary tumors, which spread from the pleura through the diaphragm and invaded the peritoneal cavity. The macroscopic characteristics of all of these tumors—pleural, pericardial, and peritoneal—were reminiscent of mesotheliomas.

All of the 43 hamsters injected with wt SV40 (wt 776 and wt 830) developed tumors. Thirty of these tumors had macroscopic characteristics of diffuse malignant mesotheliomas (Table 1). Mesotheliomas developed 3 to 6 months after virus injection, and

Table 1. *Incidence of Mesotheliomas in Syrian Hamsters Injected with Wild Type SV40 and SV40 Small t Deletion Mutants*

Virus injected	Route of injection*		
	Intracardiac	Intrapleural	Intraperitoneal
wt 776	2/5 (40%)	5/5 (100%)	4/6 (67%)
wt 830	13/21 (62%)	6/6 (100%)	N.D.
dl 883	0/12 (0%)	N.D.	N.D.
dl 2006	1/22 (5%)	N.D.	N.D.
Control†	0/20 (0%)	0/4 (0%)	0/4 (0%)

* The numbers reported show the number of animals that developed mesotheliomas/number of animals injected with the virus. The percentage of animals that developed mesotheliomas is shown in parentheses.

† Control animals were injected with the same media used to dilute the viruses. N.D. = not done.

hamsters with mesothelioma did not develop additional tumors. Lymphomas (10 hamsters), osteosarcomas (four), and myxoma (one) were observed in those hamsters that did not develop mesotheliomas. These tumors had a latency similar to mesotheliomas (3 to 6 months); two hamsters with lymphoma also developed osteosarcoma of the knee.

All of the hamsters injected ic with SV40 small t mutants dl 883 and dl 2006 developed multiple, soft, white/grey encapsulated tumors of about 2 mm to 3 cm in diameter, apparently originating from the abdominal lymph nodes. Characterization of these tumors indicated that they were either true histiocytic lymphomas or B-cell lymphomas.[23] Only one mesothelioma was observed in hamsters injected with SV40 small t mutants (Table 1). This animal, injected with dl 2006, developed both a mesothelioma and a lymphoma. None of the hamsters injected with media alone (control groups) developed any type of tumor.

Microscopic Characteristics of SV40-Induced Mesotheliomas

Histologically, the mesotheliomas revealed various morphologies. These were independent of the type of virus injected or route of virus injection. In most tumors, malignant cells formed papillary structures, the small papillae consisting of a core of delicate connective tissue covered with closely packed cuboidal cells (Figure 1A). In other tumors, or in different areas of the same tumor, neoplastic cells formed tubules or glandlike spaces (pseudoacini, Figure 1B). Some mesotheliomas were instead highly cellular, formed by interwoven bundles of spindle cells, often in mitosis, with ovoid basophilic nuclei and eosinophilic cytoplasm (Figure 1C). Thus, histologically these lesions were reminiscent either of the epithelial mesotheliomas (Figures 1, A and B) or of the spindle cell (sarcomatoid) mesotheliomas (Figure 1C) found in humans. However, most of the SV40-induced mesotheliomas were of the mixed type. In these mixed type mesotheliomas, both spindle-cell (sarcomatoid) areas and epithelial areas were observed. In one mesothelioma, areas of osseous and cartilaginous metaplasia were encountered.

To carry out *in vitro* studies on the mesothelioma cells induced by the various viruses, we used fresh tumor material to establish cultures from 17 different mesotheliomas. Mesothelioma-derived cell lines grew in tissue culture adherent to the plastic dishes. These cells showed a polygonal shape with large nuclei and abundant cytoplasm (Figure 1D).

Identification of Pleural, Pericardial, and Peritoneal Tumors as Mesotheliomas

Macroscopic and microscopic characteristics of the pleural, pericardial, and peritoneal tumors observed in these hamsters suggest that they all have a mesothelial origin. However, malignant mesothelioma must be carefully distinguished from metastatic carcinoma, particularly pulmonary adenocarcinoma.

We used histochemical stains that have been demonstrated to be useful for confirming the diagnosis of mesothelioma.[1,24] The hamster mesotheliomas produced hyaluronic acid, which was demonstrated by the Alcian blue stain. The Alcian blue stain was almost completely removed by pretreatment with hyaluronidase, demonstrating the specificity of the reaction. In addition, the absence of mucicarmine-positive and periodic acid-Schiff–positive material in the cytoplasm of these tumor cells strongly favors a diagnosis of mesothelioma over that of adenocarcinoma. These histochemical stains were performed on all of the hamster mesotheliomas observed in this study. Areas of tumors showing papillary formation also revealed occasional cells or groups of cells that were periodic acid-Schiff–positive. However, predigestion of the tissue with hyaluronidase either abolished or greatly reduced this positivity, suggesting that hyaluronic acid was the substance responsible for this staining reaction.[24] We also investigated the expression of cytokeratins in one of these tumors and its derived cell line (H9A) using a rabbit polyclonal antibody and the immunoperoxidase reaction (see Materials and Methods). Both exhibited an even stain for cytokeratins throughout the cytoplasm.

Electronmicroscopic examination can also aid in differentiating mesotheliomas from other tumors. The principal organelles stated to be useful in the diagnosis of mesothelioma include microvilli (the occasional branching character of the microvilli in mesotheliomas and the absence of core filaments are useful in distinguishing this tumor from adenocarcinoma), intercellular junctions (desmosomes and zonulae occludens), limited cytoplasmic organelles (especially rough endoplasmic reticulum), perinuclear tonofilaments, basal lamina, and intracellular lumens containing microvilli.[25,26] All of these characteristics were present in three mesotheliomas and two mesothelioma cell lines that we examined with the transmission electron microscope. Some of these ultramicroscopic characteristics are shown in Figure 2. These studies further suggest the mesothelial origin of the tumors. They also suggest that the tumor-derived cell lines were formed by cells

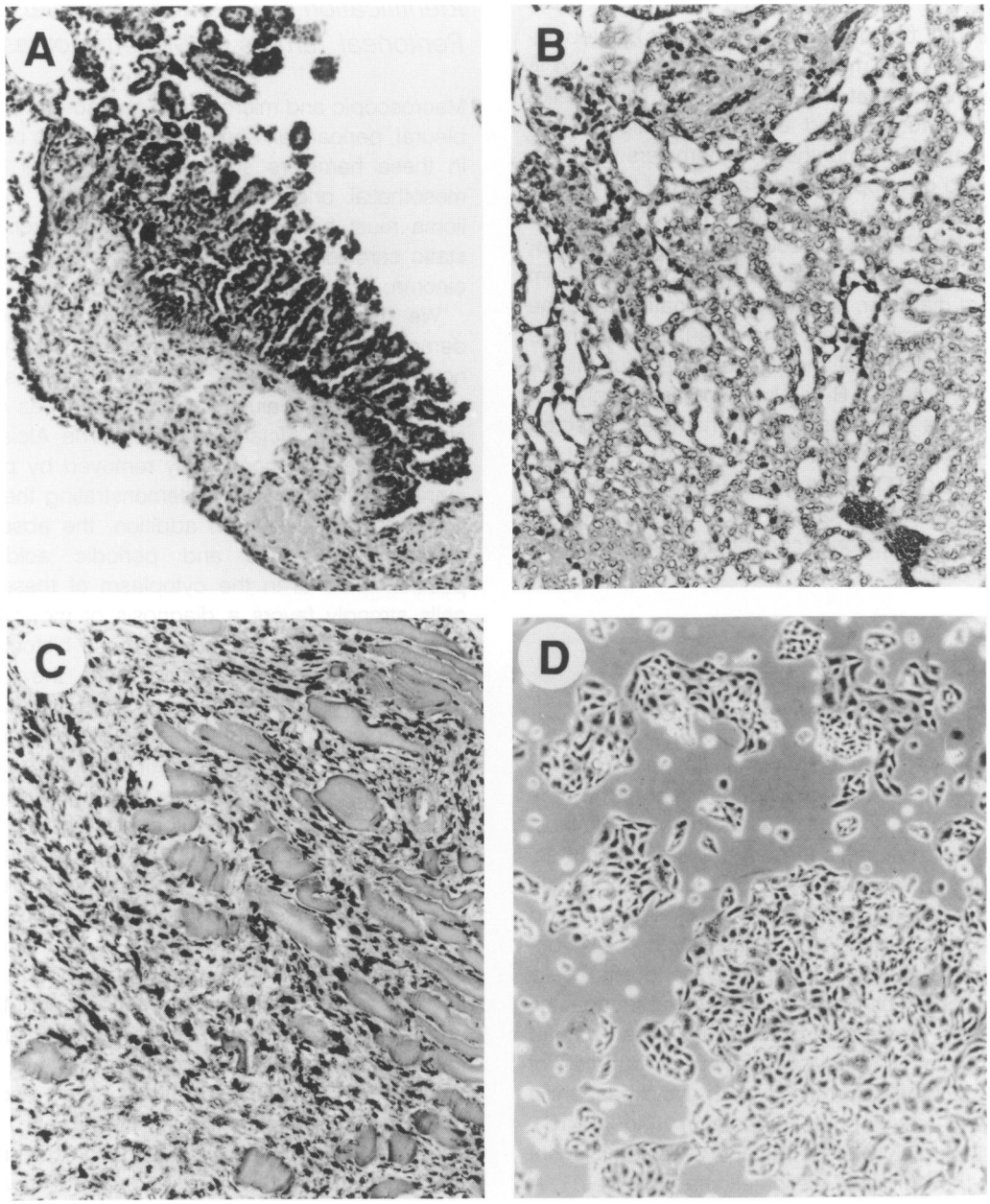

Figure 1. *Representative microscopic characteristics of SV40-induced mesotheliomas.* A: *Epithelial mesothelioma and papillary formations;* B: *epithelial mesothelioma forming tubules or glandlike spaces (pseudoacini);* C: *spindle-cell mesothelioma infiltrating muscle tissue;* D: *morphology of mesothelioma-derived cells in tissue culture. (Hematoxylin and eosin; original magnifications: ×-200).*

representative of the original tumor, as both the tumor and the derived cells had similar ultramicroscopic characteristics (Figure 2).

SV40 Integration and Expression in Hamster Mesotheliomas and Mesothelioma-Derived Cell Lines

Mesotheliomas occurred only in hamsters injected with SV40 viruses: thus there was little doubt that they were virally induced. To verify this hypothesis, we isolated high-molecular-weight cellular DNA from five mesotheliomas and three mesothelioma-derived cell lines and performed Southern blot analyses on all of them using different restriction enzymes. The *Eco*RI and *Bam*HI restriction enzymes recognize a sequence found only once in the SV40 genome. Digestion of tumor samples and derived cell lines using these enzymes indicated that the SV40 genome was integrated into the cellular DNA

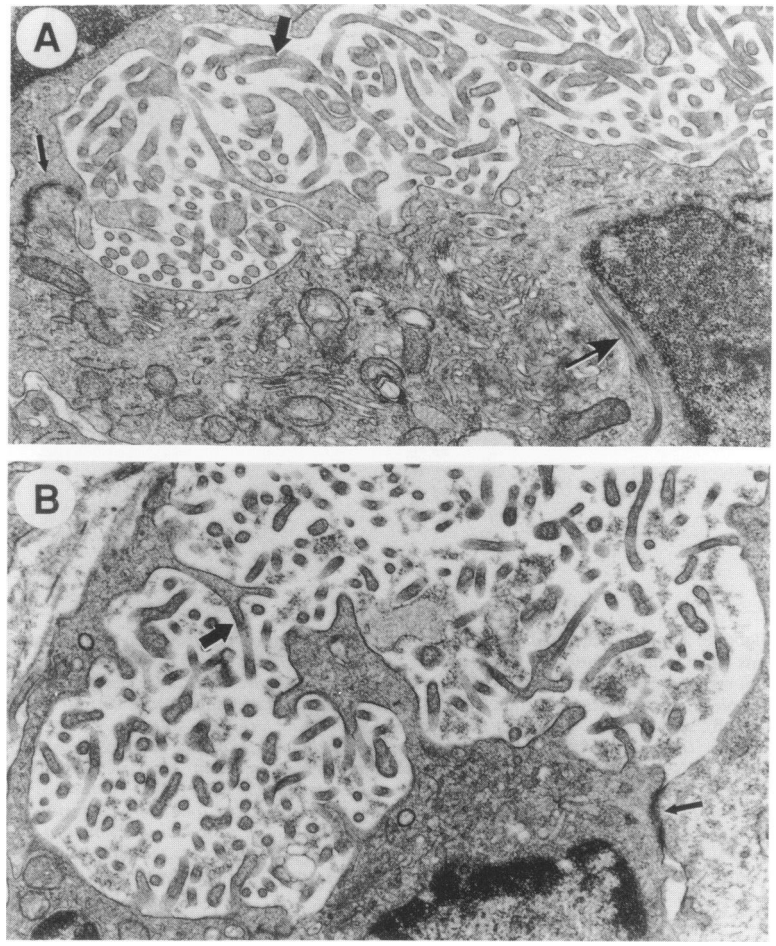

Figure 2. *Representative electronmicroscopic characteristics of mesotheliomas and derived cell lines.* **A:** *hamster pleural mesothelioma induced by the intracardiac injection of wt SV40 776 (sample H9A);* **B:** *electronmicroscopic characteristics of the mesothelioma cell line derived from the tumor shown in* **A**. *Both the original tumor and the derived cell line show ultramicroscopic characteristics of mesothelial cells. Long thin arrow points to perinuclear tonofilaments; short thin arrow points to a desmosome; thick arrow points to long branching microvilli. (Original magnification: ×24,000).*

(Figure 3A). This interpretation is based on the finding of several high-molecular-weight DNA fragments that hybridized with the SV40 probe. This experiment also suggested that the integration of SV40 in the mesotheliomas and in the derived cell lines was similar. However, in DNAs derived from these cells, we observed both the appearance of new bands and the disappearance of others originally present in the tumor (Figure 3A). Thus, either the original tumor was not clonal and a specific subpopulation/s of mesothelioma cells was selected in tissue culture, or further rearrangements of the integrated SV40 DNA occurred during the establishment and the passage of the mesothelioma-derived cells.

The use of *Eco*RI and *Bam*HI restriction enzymes is also useful for detecting the presence in transformed cells of tandem copies of SV40 DNA. These enzymes should cleave within repetitious viral sequences to free from them a unit-length copy of the viral genome that will migrate through agarose gels at the same rate as intact linear SV40 DNA (5.2kb). The presence of tandem repeats of SV40 in mesotheliomas and derived cell lines was suggested by the appearance of a prominent 5.2-kb band in most of the DNAs examined (Figure 3A). Alternatively, the presence of the 5.2-kb band might result from the presence of unintegrated SV40. To distinguish between these two possibilities, we digested the same DNAs shown in Figure 3A with *Bgl*II and *Xho*I restriction enzymes and hybridized with the SV40 probe. These restriction enzymes were chosen because they do not cut SV40 DNA. The results of these experiments suggested that all of the SV40 was integrated in the cellular genome as the SV40 probe hybridized only with high-molecular-weight DNA, and no bands corresponding to approximately 2.8 kb, the size where circular forms of SV40 migrate, were detected (Figure 3B). To confirm this interpretation, we isolated low-molecular-weight DNAs from mesothelioma-derived cells using the Hirt procedure.[27] Hybridization of these DNAs also failed to demonstrate the presence of unintegrated SV40 (not shown).

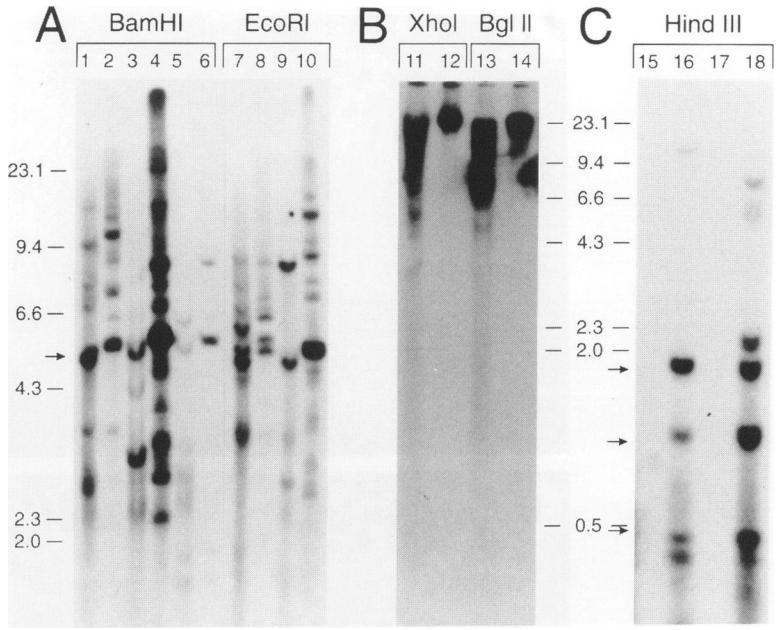

Figure 3. *Representative Southern blot analysis of SV40-induced mesotheliomas and derived cell lines. The restriction pattern shown in this figure was reproducibly observed in three separate experiments. DNAs (10 ug) were digested with:* **A:** Bam*HI* **(left)** *or* Eco*RI* **(right)** *restriction enzymes, run on 0.8% agarose gel, blotted, and hybridized with* ^{32}*P-labeled SV40 DNA. Mesothelioma* **(lanes 1** *and* **7)** *and derived cell line* **(lanes 2** *and* **8),** *sample H9A; mesothelioma* **(lanes 3** *and* **9)** *and derived cell line* **(lanes 4** *and* **10),** *sample H2A; and mesothelioma* **(lane 5)** *and derived cell line* **(lane 6),** *sample H25A. The arrow points to the 5.2-kb linear form of SV40 DNA resulting from the presence of tandem repeats of SV40 DNA in the genomic DNA. Some of the DNA fragments run slightly differently in some lanes because of differences in DNA viscosity between the tumor and the cellular DNAs. These differences were reproducibly observed in several experiments and could not be eliminated by further purifications of the DNAs. Sizes are in kilobases.* **B:** *DNAs from mesothelioma sample H2A* **(lanes 11** *and* **13)** *and derived cell line* **(lanes 12** *and* **14),** *digested with* Xho*I and* Bgl*II restriction enzymes, run on 0.8% agarose gel, blotted, and hybridized with* ^{32}*P-labeled SV40 DNA.* **C:** *DNAs obtained from mesotheliomas H2A* **(lane 16)** *and H9A* **(lane 18),** *digested with* Hind*III restriction enzyme, run on 0.8% agarose gel, blotted, and hybridized with* ^{32}*P-labeled SV40 DNA. The 1.7-, 1.1-, and 0.5-kb fragments corresponding to the early region of SV40 are indicated by arrows.* **Lanes 15** *and* **17** *are control DNAs from liver and heart from hamsters H2A and H9A, respectively.*

We further analyzed the status of the SV40 genome in the mesotheliomas and in the derived cell lines by digesting these DNAs with *Hind*III which cuts the SV40 DNA at multiple sites. Following hybridization of these *Hind*III-digested DNAs with a ^{32}P-labeled SV40-DNA probe, we detected the 1.7-, 1.1-, and 0.5-kb SV40 internal fragments that correspond to the early region of the virus (Figure 3C). The SV40 early region codes for the large T and small t tumor antigens, which are responsible for SV40 transformation and oncogenicity. Finally, these experiments indicated that DNAs extracted from the heart and the liver of the five mesothelioma DNA samples studied did not contain detectable SV40 DNA sequences (Figure 3C). We conclude from these experiments that 1) SV40 is integrated in the mesotheliomas and derived cell lines and 2) SV40 is not detectable in the normal tissue of hamsters bearing mesotheliomas. These experiments also suggest that SV40 is integrated in one or more sites (depending on the tumor) in the cellular genome; that in some of these sites tandem repeats of SV40 DNA exist; that the early region of SV40 is integrated; and that none of these DNAs seems to contain unit length-free SV40 DNA sequences.

We next investigated the expression of the viral genome in seven different mesothelioma-derived cell lines by immunoprecipitation studies. All of the seven cell lines studied expressed SV40 T antigens (Figure 4). Finally, we investigated whether mesothelioma-derived cells uniformly expressed T antigen or whether they were formed by a mixed cell population some of which were T antigen negative. Almost 100% of the cells of each of these seven mesothelioma lines contained nuclear SV40 T antigen that was detected by immunofluorescence and immunohistochemical studies (not shown).

Discussion

The data from this study indicate that SV40, injected ic, ipl, or ipt, induces diffuse malignant mesotheliomas in Syrian hamsters. Mesotheliomas were identified by their macroscopic, microscopic, ultramicroscopic, and histochemical characteristics. These

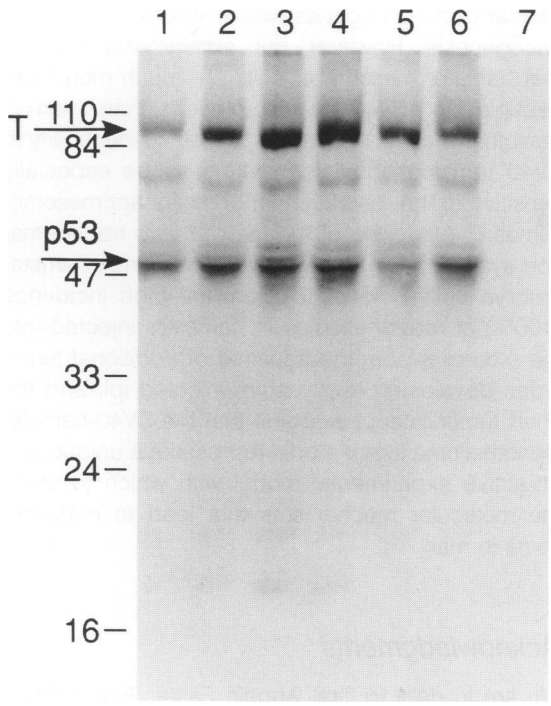

Figure 4. *Representative immunoprecipitation analysis of ^{35}S-labeled lysates from SV40-induced mesothelioma-derived cell lines. Lysates were immunoprecipitated with the monoclonal antibody Pab 419, which recognizes both SV40 large T and SV40 small t antigens.* **Lanes 1** *to* **6** *are lysates obtained from cell lines derived from different mesotheliomas.* **Lane 7** *is a control (lysate from* **lane 1**) *immunoprecipitated with normal mouse IgG. The positions of the SV40 large T antigen and the cellular anti-oncogene product p53 are indicated by arrows. Further exposure of this same radiogram resulted in the appearance of the small t antigen band at the expected molecular weight of 19 kd.*

characteristics were remarkably similar to those observed in mesotheliomas of humans. SV40-induced hamster mesotheliomas spread along the pleural, pericardial, and/or peritoneal surfaces, obliterating their cavities and infiltrating the diaphragm and the chest wall in the absence of distant metastases. Histologically, epithelial, spindle-cell, and more often mixed type mesotheliomas were observed. Ultramicroscopically, mesotheliomas and derived cell lines showed long branching microvilli without core filaments, basal lamina, intracellular lumens, perinuclear tonofilaments, intercellular junctions, absence of secretory granules, and limited cytoplasmic organelles, especially rough endoplasmic reticulum. Finally, these mesotheliomas produced hyaluronic acid as indicated by the strong reactivity of Alcian blue staining, contained cytokeratins, but they were negative with the mucicarmine and periodic acid-Schiff stains. On the basis of these data, we identify these tumors and derived cell lines as mesotheliomas and mesothelioma-derived cell lines, respectively. In humans, mesotheliomas must be carefully distinguished from metastatic adenocarcinomas. The characteristics of the SV40 hamster tumors listed above favor a diagnosis of mesothelioma rather than that of carcinoma. Furthermore, the origin of these tumors in hamsters was obvious, because the numerous tumors at different stages of development afforded ample opportunity for tracing their growth. Finally, we did not observe carcinomas, at any site, in any of the animals inoculated with SV40.

All of the animals injected ipl, with two different strains of wt SV40 (wt 776 and wt 830), developed mesotheliomas. When SV40 was injected ic or ipt, more than 50% of hamsters developed mesotheliomas. SV40 DNA sequences were detected by Southern blot hybridization in DNAs extracted from these mesotheliomas. Cell lines derived from these tumors both contained and expressed the SV40 early region of DNA, as detected by Southern blot and immunoprecipitation studies. In addition, 100% of the cells from each of the seven mesothelioma-derived cell lines studied, as detected by immunofluorescence and immunohistochemical studies, expressed SV40 T antigens. Thus there is no doubt that mesotheliomas were induced by the SV40 that had been injected into these hamsters. Furthermore, the absence of spontaneous mesotheliomas in control animals injected with media alone and the rarity of mesotheliomas in hamsters injected with SV40 small t mutants (one animal developed mesothelioma out of 34 hamsters injected) further suggest that the SV40 virus played a causal role in the induction of these mesotheliomas and that the small t antigen of SV40 was required in this process.

The specific routes of SV40 inoculation used in our studies apparently played a key role in the induction of hamster mesotheliomas. In fact, in previous studies mesotheliomas were not observed following subcutaneous,[7] intracerebral,[8] or intravenous[10] inoculation of SV40. However, Lipotich and colleagues reported utilizing a cell line (800TU) derived from one hamster mesothelioma induced by SV40.[28] This mesothelioma apparently developed accidentally in experiments in which newborn hamsters were injected subcutaneously between the scapulae with SV40. All the other animals in those experiments developed *in situ* fibrosarcomas (Moyer RC, personal communication). It is possible that in one of those subcutaneous injections, the needle reached the pleura and induced that single hamster mesothelioma.

The involvement of small t in mesothelial cell transformation is intriguing. The small t antigen of SV40 is required in tissue culture for transformation of resting cells; when cells are cycling its function is dispensable.[11,19,29] *In vivo*, SV40 small t deletion

mutants have a restricted oncogenicity, and they almost exclusively induce B lymphomas and true histiocytic lymphomas.[16–23] The biochemical activities of the small t antigen responsible for its biological effects are still unclear. Recently, it has been shown that, *in vitro*, the small t antigen binds[30,31] and inhibits[32–34] the activity of the cellular phosphatase 2A. It has been shown that, *in vitro*, the inhibitory action of small t on phosphatase 2A prevents dephosphorylation of the large T antigen of SV40 and the product of the cellular anti-oncogene p53.[33] Thus the inhibitory activity of small t may result in an alteration of the phosphorylation state of cellular and viral proteins affecting DNA replication.[34] The activity of these proteins is carefully regulated by phosphorylation and dephosphorylation events. It is possible that in addition to physical binding between the large T antigen of SV40 and the products of the cellular anti-oncogenes p53 and RB (which seems to play a key role in the process of SV40 transformation[12,13]), alteration of the phosphorylation state of RB and p53 proteins by the small t antigen may be required to inactivate completely their function and allow the cell to progress to the S phase of the cell cycle during which transformation occurs.[12,13] Thus, mesothelial cells may have a very low cycling rate, making transformation in this system dependent on small t.

Thirty years ago Gerber and Kirschstein[8] reported that SV40 induced ependymomas in hamsters. Very recently Bergsagel and colleagues[35] reported that human ependymomas also contain and express SV40 sequences, suggesting that SV40 has an etiologic role in these human tumors. These results indicate the relevance that the SV40-hamster tumor model may have for understanding some of the events responsible for human cancer, including the identification of those human tumors (ependymomas) that may be SV40-induced. We report here that SV40 induces mesotheliomas in hamsters: it will clearly be of interest to examine human mesotheliomas for SV40 DNA sequences. However, it is important to consider that SV40 per se seems unable fully to transform human mesothelial cells. For example, SV40 has the capacity to immortalize, *in vitro*, human mesothelial cells,[36] but these cells do not induce tumors in nude mice.[36] Transfection of these cells with the *ras* oncogene renders them oncogenic,[37] suggesting that in addition to the expression of the SV40 tumor antigens, alterations of key cellular proto-oncogenes are required to fully transform human mesothelial cells. This does not exclude the possibility that, in some instances, the random chromosomal changes induced by the SV40 large T antigen[13] may result in alterations of cellular proto-oncogenes, which in turn may lead to oncogenesis. However, our experiments indicate that hamster mesothelial cells are much more susceptible to SV40 transformation than human mesothelial cells. In addition to their susceptibility to SV40 transformation, hamsters may be especially sensitive to the development of malignant mesotheliomas.[38] Also, one of the best *in vitro* transformation systems for mineral fibers uses Syrian hamster embryo cells.[39] In conclusion, the high incidence (100%) of mesotheliomas in hamsters injected into the pleural space, the absence of additional tumor types developing in hamsters injected ipl, and the short tumor latency suggest that the SV40-hamster mesothelioma tumor model represents a unique and attractive experimental model with which to study the molecular mechanisms that lead to mesothelioma in man.

Acknowledgments

We are in debt to Drs. Angelo Festa, Torgny Fredrickson, Marie C. Jaurand, Annabel Liebilt, Antonio Procopio, Harold L. Stewart, Maria D. Tocco, and William Travis for reviewing some of the histology of the mesotheliomas; Drs. Arthur S. Levine, William R. Sanslone, and Harold L. Stewart, for critically reviewing this manuscript; and Drs. Sisir K. Chattopadhyay and Kathleen Dixon for help and advice with various methods.

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